

# **UCLA**

## **UCLA Previously Published Works**

### **Title**

ASXL2 regulates hematopoiesis in mice and its deficiency promotes myeloid expansion.

### **Permalink**

<https://escholarship.org/uc/item/5j91t9wn>

### **Journal**

Haematologica, 103(12)

### **ISSN**

0390-6078

### **Authors**

Madan, Vikas  
Han, Lin  
Hattori, Norimichi  
et al.

### **Publication Date**

2018-12-01

### **DOI**

10.3324/haematol.2018.189928

Peer reviewed



EUROPEAN  
HEMATOLOGY  
ASSOCIATION



Ferrata Storti  
Foundation

# ASXL2 regulates hematopoiesis in mice and its deficiency promotes myeloid expansion

Vikas Madan,<sup>1</sup> Lin Han,<sup>1,2</sup> Norimichi Hattori,<sup>1,3</sup> Weoi Woon Teoh,<sup>1</sup>  
Anand Mayakonda,<sup>1</sup> Qiao-Yang Sun,<sup>1</sup> Ling-Wen Ding,<sup>1</sup>  
Hazimah Binte Mohd Nordin,<sup>1</sup> Su Lin Lim,<sup>1</sup> Pavithra Shyamsunder,<sup>1</sup>  
Pushkar Dakle,<sup>1</sup> Janani Sundaresan,<sup>1</sup> Ngan B. Doan,<sup>4</sup> Masashi Sanada,<sup>5,6</sup>  
Aiko Sato-Otsubo,<sup>6</sup> Manja Meggendorfer,<sup>7</sup> Henry Yang,<sup>1</sup> Jonathan W. Said,<sup>4</sup>  
Seishi Ogawa,<sup>6</sup> Torsten Haferlach,<sup>7</sup> Der-Cherng Liang,<sup>8</sup> Lee-Yung Shih,<sup>9</sup>  
Tsuyoshi Nakamaki,<sup>3</sup> Q. Tian Wang<sup>10</sup> and H. Phillip Koeffler<sup>1,11,12</sup>

**Haematologica** 2018  
Volume 103(12):1980-1990

<sup>1</sup>Cancer Science Institute of Singapore, National University of Singapore; <sup>2</sup>Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore; <sup>3</sup>Division of Hematology, Department of Medicine, School of Medicine, Showa University, Shinagawa-Ku, Tokyo, Japan; <sup>4</sup>Department of Pathology and Laboratory Medicine, Santa Monica-University of California-Los Angeles Medical Center, Los Angeles, CA, USA; <sup>5</sup>Department of Advanced Diagnosis, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Japan; <sup>6</sup>Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Japan; <sup>7</sup>MLL Munich Leukemia Laboratory, Germany; <sup>8</sup>Division of Pediatric Hematology-Oncology, Mackay Memorial Hospital and Mackay Medical College, Taipei, Taiwan; <sup>9</sup>Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan, Taiwan; <sup>10</sup>Department of Biological Sciences, University of Illinois at Chicago, IL, USA; <sup>11</sup>Cedars-Sinai Medical Center, Division of Hematology/Oncology, UCLA School of Medicine, Los Angeles, CA, USA and <sup>12</sup>Department of Hematology-Oncology, National University Cancer Institute of Singapore (NCIS), National University Hospital, Singapore

LH and NH contributed equally to this work

## ABSTRACT

Chromosomal translocation t(8;21)(q22;q22) which leads to the generation of oncogenic RUNX1-RUNX1T1 (AML1-ETO) fusion is observed in approximately 10% of acute myelogenous leukemia (AML). To identify somatic mutations that co-operate with t(8;21)-driven leukemia, we performed whole and targeted exome sequencing of an Asian cohort at diagnosis and relapse. We identified high frequency of truncating alterations in ASXL2 along with recurrent mutations of *KIT*, *TET2*, *MGA*, *FLT3*, and *DHX15* in this subtype of AML. To investigate in depth the role of ASXL2 in normal hematopoiesis, we utilized a mouse model of ASXL2 deficiency. Loss of ASXL2 caused progressive hematopoietic defects characterized by myeloid hyperplasia, splenomegaly, extramedullary hematopoiesis, and poor reconstitution ability in transplantation models. Parallel analyses of young and >1-year old *Asxl2*-deficient mice revealed age-dependent perturbations affecting, not only myeloid and erythroid differentiation, but also maturation of lymphoid cells. Overall, these findings establish a critical role for ASXL2 in maintaining steady state hematopoiesis, and provide insights into how its loss primes the expansion of myeloid cells.

## Correspondence:

csivm@nus.edu.sg or  
nhattor@med.showa-u.ac.jp or  
sly7012@cgmh.org.tw

Received: January 31, 2018.

Accepted: July 26, 2018.

Pre-published: August 9, 2018.

doi:10.3324/haematol.2018.189928

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: [www.haematologica.org/content/103/12/1980](http://www.haematologica.org/content/103/12/1980)

©2018 Ferrata Storti Foundation

Material published in *Haematologica* is covered by copyright. All rights are reserved to the Ferrata Storti Foundation. Use of published material is allowed under the following terms and conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>. Copies of published material are allowed for personal or internal use. Sharing published material for non-commercial purposes is subject to the following conditions: <https://creativecommons.org/licenses/by-nc/4.0/legalcode>, sect. 3. Reproducing and sharing published material for commercial purposes is not allowed without permission in writing from the publisher.



## Introduction

Chromosomal translocation t(8;21)(q22;q22) is a frequent cytogenetic abnormality observed in approximately 10% of acute myelogenous leukemia (AML). This rearrangement involves *RUNX1* gene on chromosome 21 and *RUNX1T1* gene on chromosome 8 and results in generation of oncogenic RUNX1-RUNX1T1 fusion protein (also known as AML1-ETO).<sup>1</sup> However, expression of RUNX1-RUNX1T1 alone, either as a transgene or using viral transduction in hematopoietic cells, is insufficient to cause leukemia in mice.<sup>2-4</sup> Loss of sex chromosome and other chromosomal aberrations, as well as recurrent somatic mutations of *KIT*, *FLT3*, *NRAS* and *KRAS*, are associated with t(8;21) AML.<sup>2,4</sup> Recent high-throughput sequencing

approach has identified additional mutagenic events involving *ASXL2*, *ASXL1* and *DHX15* genes in t(8;21) AML.<sup>5-10</sup> Several secondary genetic events that co-operate with RUNX1-RUNX1T1 fusion in inducing leukemia have been demonstrated using mouse models.<sup>2-4,11</sup>

ASXL1, ASXL2 and ASXL3 are human homologs of *Drosophila* Asx (Additional sex combs) and function as epigenetic regulators through recruitment of Polycomb group repressor complexes (PRC) and Trithorax group activator complexes.<sup>12,13</sup> ASXL proteins can interact with BAP1, NCOA1, EZH2, WTIP and nuclear receptors, which suggests diverse functions of ASXL family members in epigenetic and transcriptional regulation.<sup>12,13</sup> Somatic mutations of *ASXL1* occur in a range of hematologic disorders,<sup>14-20</sup> and its deletion in mouse hematopoietic cells results in multilineage cytopenias and dysplasia, which are associated with global reduction of H3K27 trimethylation.<sup>21</sup> In contrast, somatic mutations of *ASXL2* are observed exclusively in t(8;21) AML,<sup>5,8</sup> while mutations of *ASXL3* are not reported in hematologic malignancies.<sup>13</sup> Silencing of *ASXL2* is partially embryonic lethal depending on the genetic background, and leads to congenital heart defects. Surviving homozygous *Asxl2* mutant mice are smaller than wild-type littermates, demonstrate skeletal homeotic transformations consistent with disruption of Polycomb/Trithorax complex functions, develop cardiac dysfunction, and exhibit decreased bone mineral density.<sup>22-26</sup> More recently, its role in normal hematopoietic development and leukemic development has been investigated.<sup>27,28</sup>

In the present study, we used exome sequencing to identify somatic mutations co-operating with RUNX1-RUNX1T1 rearrangement in AML. Frequent truncating mutations of *ASXL2* were identified in our t(8;21) AML cohort. Using an *Asxl2*-deficient mouse model, we aimed to clarify the function of ASXL2 in hematopoiesis. We demonstrate that ASXL2 is crucial to maintain hematopoietic stem cell (HSC) number and function. Loss of ASXL2 leads to myeloproliferation and extramedullary hematopoiesis, as well as to progressive defects in multilineage differentiation. These results establish ASXL2 as a key component of the epigenetic machinery involved in maintaining hematopoietic development.

## Methods

### Acute myelogenous leukemia samples

t(8;21) AML was diagnosed by karyotypic analysis and/or reverse transcriptase PCR assay for the detection of fusion transcript of RUNX1-RUNX1T1. Samples were collected with informed consent at diagnosis, during complete remission, and at time of frank relapse in accordance with the Declaration of Helsinki. Bone marrow (BM) mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation (1.077 g/mL; Amersham Pharmacia, Sweden). The study was approved by the institutional review boards of the respective institutes.

### Exome sequencing and somatic variant discovery

DNA was sheared using a Covaris instrument and assessed on a 2100 Bioanalyzer. Library preparation and exome sequencing were performed as described previously.<sup>29</sup> For whole exome sequencing, DNA were captured using SureSelect Human All Exon 50Mb kit (Agilent), according to the manufacturer's instructions. For targeted capture library, Agilent's SureSelect XT2 Target

Enrichment System for Illumina Multiplexed Sequencing was used. RNA baits were designed to capture exons of 530 genes (*Online Supplementary Table S1*) and libraries were sequenced on HiSeq 2000 (Illumina).

100 bp paired-end reads were aligned to human reference genome (reference build: hg19) using bwa-mem aligner with default parameters. PCR duplicate reads were marked with samblaster.<sup>30</sup> Resulting BAM files were further processed according to GTAK best practices including INDEL re-alignment and base quality recalibration ([https://software.broadinstitute.org/gatk/best-practices/bp\\_3step.php?case=GermShortWGS](https://software.broadinstitute.org/gatk/best-practices/bp_3step.php?case=GermShortWGS)). Somatic variants were detected using Varscan2 somatic command.<sup>31</sup> Raw variants were processed with processSomatic command with the *P*-value set to 0.05 to obtain high confident variants. Variants were further processed using fpFilter per script to remove potential false positives (<https://github.com/ckandoth/variant-filter>). Resulting variants were annotated using Variant Effect Predictor and filtered against germline variants in both dbSNP and ExAC, while keeping deleterious and clinically significant variants.<sup>32</sup> For samples without germline controls, variants were called using MuTect using a panel of normal derived from in-house cohort of AML remission samples, as described previously.<sup>29,33</sup> Oncoplots were drawn using maftools Bioconductor package.<sup>34</sup> All the somatic mutations for whole exome sequencing and about 90% mutations reported for targeted exome sequencing were validated using PCR amplification and Sanger sequencing.

## Mice

Mice with *Asxl2* gene-trap allele [referred to as knockout (KO) allele in this study] have been described previously.<sup>24</sup> *Asxl2* heterozygous KO mice were maintained on C57BL/6J (B6) and 129Sv (129) genetic backgrounds. *Asxl2* homozygous null mice [and corresponding wild-type (WT) controls] used in all experiments were B6 x 129 F1 obtained by crossing *Asxl2* heterozygous B6 and 129 mice. Mouse colonies were housed and maintained at the animal facility of Comparative Medicine Centre, National University of Singapore (NUS). All mice experiments were approved by Institutional Animal Care and Use Committee, NUS, Singapore.

## Flow cytometry

Cells were incubated with fluorochrome-conjugated antibodies for 30 min on ice, washed and resuspended in SYTOX Blue Dead Cell Stain (ThermoFisher Scientific) before acquisition on FACS LSR II flow cytometer (BD Biosciences). Sorting of cells was performed on FACSARIA cell sorter (BD Biosciences) and data were analyzed using FACSDIVA software (BD Biosciences). See *Online Supplementary Table S2* for the list of antibodies used for flow cytometric staining.

## Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 software.

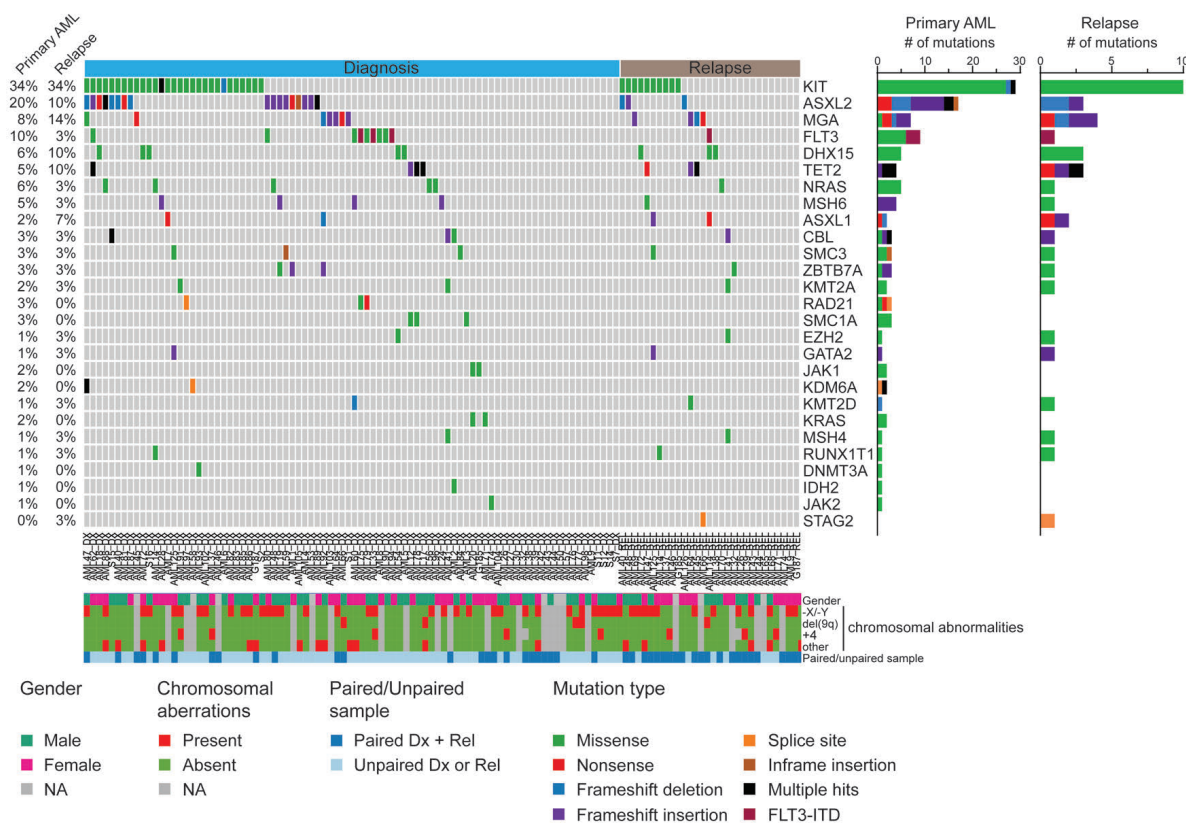
## Accession numbers

The accession numbers for the sequencing data reported in this study are SRP122878 (exome sequencing) and GSE106798 (RNA sequencing).

## Results

### Mutational profile of RUNX1-RUNX1T1 AML

We performed whole-exome sequencing of 10 paired samples of newly-diagnosed and relapsed AML with RUNX1-RUNX1T1 rearrangement from an Asian cohort,



**Figure 1. Mutational landscape of t(8;21) acute myelogenous leukemia (AML).** Matrix displays individual somatic mutations detected in diagnosis and relapse AML with RUNX1-RUNX1T1 fusion. Mutational frequencies are illustrated on the left and bar graphs on the right depict absolute number and type of mutations. The annotation bars at the bottom display patient information including the cytogenetic aberrations detected in karyotype analysis. NA: information not available.

along with matched germline (complete remission) DNA (Online Supplementary Table S3). We achieved a mean depth of 100x (range 64-271x); an average 68% of nucleotides were covered by at least 20 reads (Online Supplementary Figure S4). We identified 55 somatic mutations (in 52 genes) in newly-diagnosed cases and 76 mutations (in 69 genes) at relapse, which included recurrent mutations in *KIT*, *TET2*, *DHX15* and *MGA* (Online Supplementary Table S4). We assessed the stability of mutations in diagnosis and relapse samples analyzed using whole exome sequencing. Overall, the disease evolution followed the pattern reported previously for AML,<sup>35</sup> as the founding clone or a subclone at diagnosis survived the therapy, gained additional mutations, and expanded at relapse (Online Supplementary Figure S2). Interestingly, all four mutations of *TET2* (in 3 cases) were acquired at relapse (Online Supplementary Table S4).

Further to uncovering the repertoire of co-operating mutations in t(8;21) AML, we analyzed mutational status of 530 genes (Online Supplementary Table S4) in 76 newly-diagnosed and 19 relapse t(8;21) AML cases using targeted-exome sequencing (Online Supplementary Table S3). In this cohort, the mean sequencing coverage across targeted bases was 96x (range 76-319x), with 70% of bases covered greater than 20x (Online Supplementary Figure S4).

Overall, we observed that the mutations of *KIT* were most frequent at both diagnosis and relapse in our cohort, along with recurring alterations in *ASXL2*, *MGA*, *DHX15*, *TET2* and *FLT3* genes (Figure 1, Online Supplementary Table

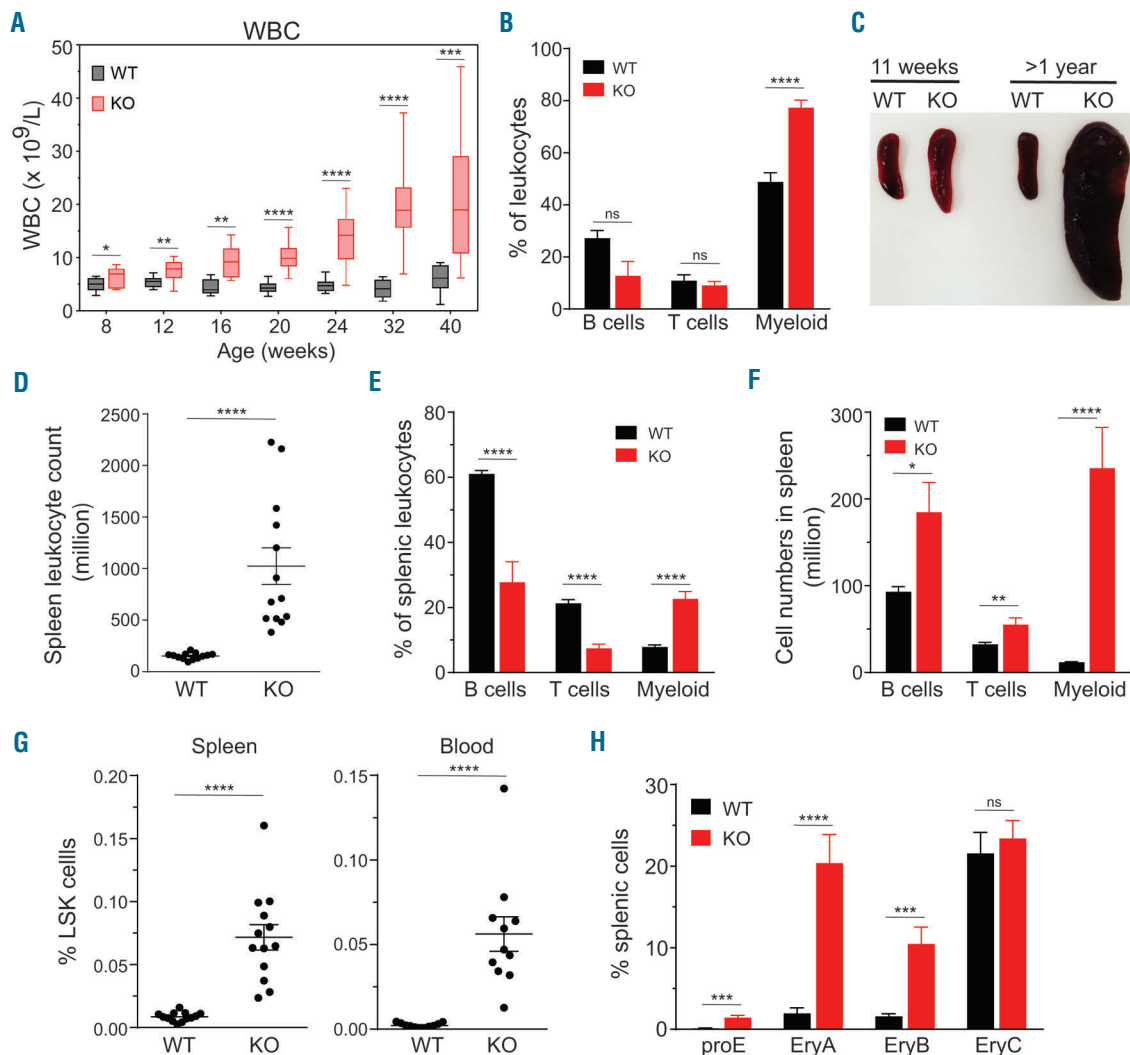
S5 and Online Supplementary Figure S3). Somatic mutations of *ASXL2* were detected in 20% of newly-diagnosed (17 of 86) and 10% of relapsed (3 of 29) cases while *ASXL1* was mutated at a much lower frequency (2% of newly-diagnosed cases and 7% of relapsed cases) (Figure 1, Online Supplementary Table S5 and Online Supplementary Figure S3). Mutations of *DHX15*, a RNA helicase, occurred exclusively at Arg222 residue in 7 cases. *MGA* and *TET2* genes predominantly harbored nonsense and frameshift mutations spread throughout the transcript (Figure 1, Online Supplementary Figure S3 and Online Supplementary Table S5).

*ASXL2* was the second most frequently mutated gene, and interestingly, all alterations were truncating mutations located in exons 11 and 12 (Figure 1 and Online Supplementary Figure S3). Unlike alterations of *ASXL1*, which are recurrent in several hematologic diseases, recent reports<sup>5,8,9</sup> have highlighted the incidence of *ASXL2* mutations specifically in the t(8;21) subtype of AML. In this study, we aimed to investigate the consequences of deficiency of *ASXL2* on hematopoietic development using a mouse model.

#### Impaired hematopoiesis in *Asxl2*-deficient mice

Our RT-PCR analysis showed that *Asxl2* is expressed in a broad range of murine hematopoietic cell types (Online Supplementary Figure S4A). To investigate the physiological role of *ASXL2* in steady-state hematopoiesis, we used genetrapped mice (referred to as *Asxl2* KO mice) described previous-



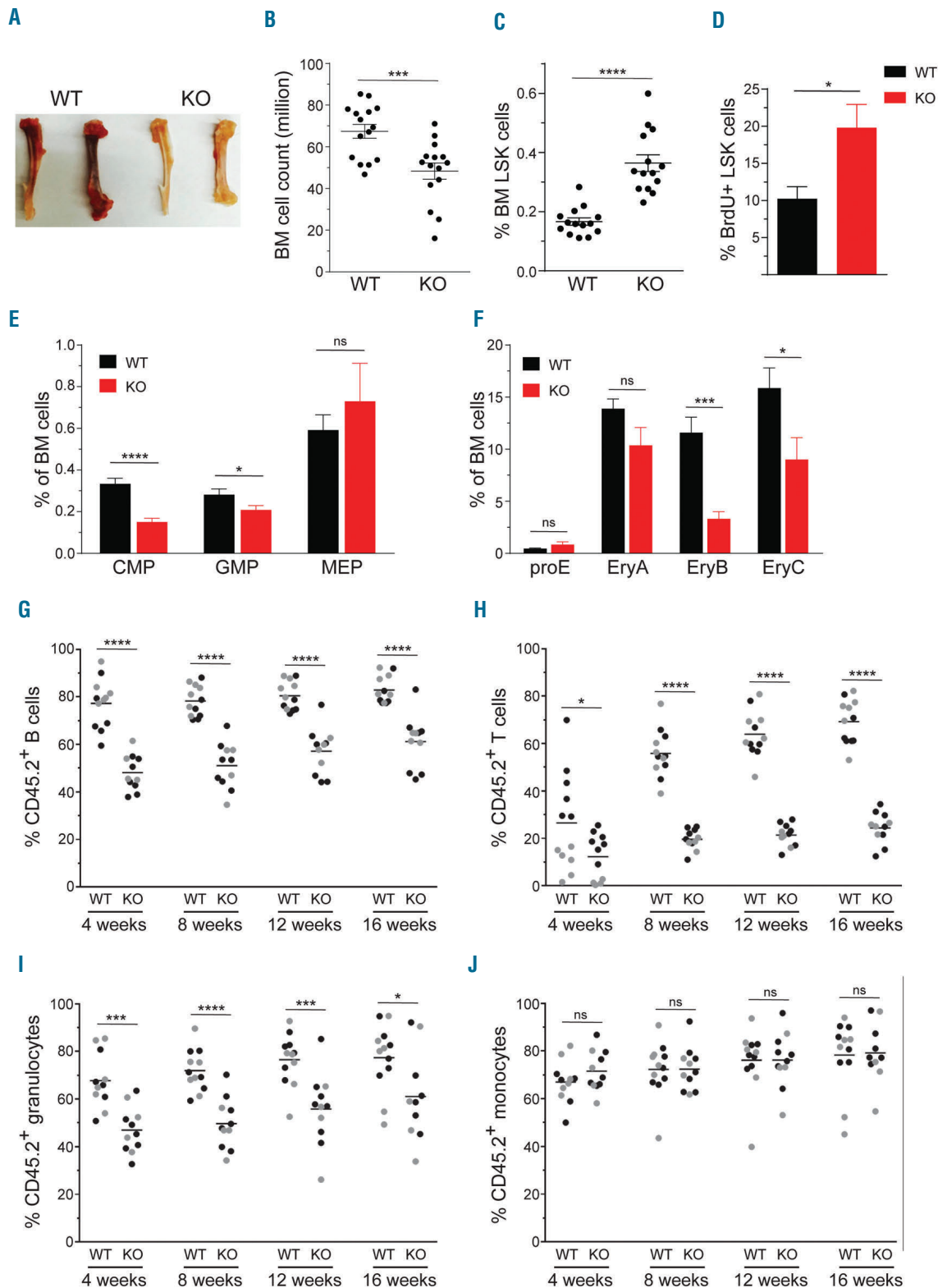


**Figure 2 Myeloproliferation and extramedullary hematopoiesis in *Asxl2*-deficient mice.** (A) White blood cell counts in peripheral blood of 8–10 week old wild-type (WT) and *Asxl2*-deficient mice. Whiskers extend from the minimum to the maximum values. (B) Proportion of B cells (CD19<sup>+</sup>), T cells (CD3<sup>+</sup>), and myeloid cells (CD11b<sup>+</sup>) in the peripheral blood of >1-year old WT and *Asxl2* knockout (KO) mice determined using flow cytometry (B and T cells, n=3; myeloid cells, n=11). (C) Representative photograph of spleens isolated from young (11-week old) and old (>1-year old) WT and *Asxl2* KO mice. (D) Total leukocyte counts in spleens from >1-year old mice. (E and F) Frequencies (E) and absolute numbers (F) of lymphoid and myeloid cells in spleens of old WT and KO mice. Flow-cytometric analysis on splenocytes was similar to (B) (n=12–13). (G) Frequency of LSK cells in spleen and peripheral blood of old WT and KO mice. (H) Proportion of erythroid progenitors detected in flow cytometric analysis (staining with CD71 and TER119 antibodies) of spleens from old WT and KO mice (n=10). Bars represent mean±Standard Error of Mean. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001, ns: not significant.

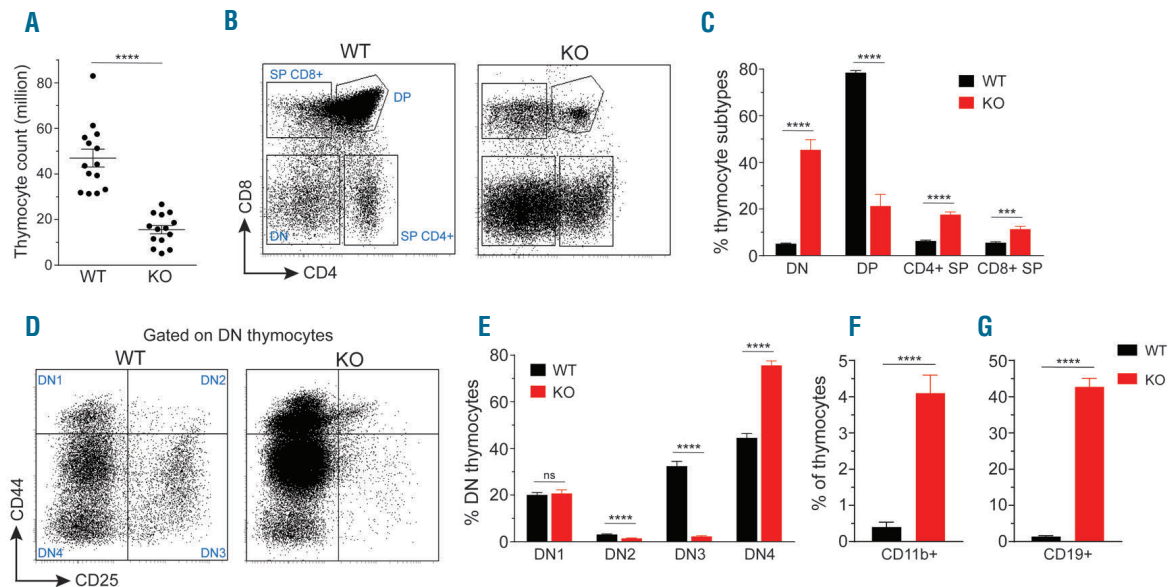
ly.<sup>24</sup> As reported,<sup>24</sup> *Asxl2* KO mice had a significantly shorter life span compared with the WT mice (*Online Supplementary Figure S4B*). We observed a significant reduction in *Asxl2* transcript and protein levels in the BM, spleen and thymus of *Asxl2* KO mice compared to WT mice (*Online Supplementary Figure S5A–F*), whereas the expression of *Asxl1* was not affected (*Online Supplementary Figure S5G–I*). We cultured the BM cells from WT and KO mice for two weeks in myeloid differentiation-promoting conditions and observed an increase in the proportion of CD11b<sup>+</sup> cells with a concomitant decrease in Lin-Kit<sup>+</sup> cells in the cultures from *Asxl2*-deficient BM, suggesting altered myeloid differentiation (*Online Supplementary Figure S6A and B*). Moreover, in re-plating assays, we observed a sustained ability of *Asxl2* KO BM cells to generate myeloid colonies compared with WT cells (*Online Supplementary Figure S6C*).

To gain initial insights into the effect of ASXL2 deficiency on hematopoiesis *in vivo*, we periodically analyzed the

peripheral blood (PB) from *Asxl2* KO and WT mice beginning at eight weeks of age. We observed an age-dependent increase in white leukocyte count in the PB of *Asxl2*-deficient mice, while the numbers were unchanged in the WT mice (Figure 2A). This was associated with reduced red blood cell (RBC) counts and hemoglobin in peripheral blood of *Asxl2* KO mice with increasing age (*Online Supplementary Figure S7*). This observation suggested progressive defects in the hematopoietic compartment in *Asxl2* KO mice, and therefore we investigated systematically hematopoietic development in >1-year old mice. Flow cytometric analysis of blood leukocytes from these mice showed a higher proportion of myeloid (CD11b<sup>+</sup>) and a reduced proportion of lymphoid (CD19<sup>+</sup> and CD3<sup>+</sup>) cells in KO mice (Figure 2B and *Online Supplementary Figure S8A*). Old *Asxl2* KO mice exhibited extensive splenomegaly (Figure 2C and D) which was associated with marked myeloproliferation evident by increased fre-



**Figure 3. ASXL2 deficiency perturbs frequency and function of hematopoietic stem cells (HSCs.)** (A) Representative photograph of tibias and femurs, respectively, from >1-year old wild-type (WT) and *Asxl2* knockout (KO) mice. (B) Bone marrow (BM) cellularity (two femurs and two tibias) in old WT and *Asxl2* KO mice. (C) Proportion of LSK cells in the BM of old mice ( $n=14$ ). (D) Frequency of BrdU+ LSK cells in the BM of 10-20-weeks old WT and *Asxl2* deficient mice ( $n=5$ ). (E) Proportions of CMP, GMP and MEP in the BM of >1-year old WT and KO mice ( $n=12$ ). (F) Frequency of erythroid precursors (proE: CD71<sup>+</sup>TER119<sup>+</sup>, EryA: CD71<sup>+</sup>TER119<sup>+</sup>FSC<sup>+</sup>, EryB: CD71<sup>+</sup>TER119<sup>+</sup>FSC<sup>+</sup> and EryC: CD71<sup>+</sup>TER119<sup>+</sup>FSC<sup>+</sup>) in BM of >1-year old WT and *Asxl2* KO mice ( $n=8$ ). (G-J) Competitive repopulation assay: 4000 (black circles) or 2000 (gray circles) LSK cells were injected in recipient mice and proportion of donor-derived cells (CD45.2<sup>+</sup>) in peripheral blood was determined at 4, 8, 12 and 16 weeks post transplantation. Ability of WT and *Asxl2* KO LSK cells to reconstitute B cells (CD19<sup>+</sup>) (G), T cells (CD3<sup>+</sup>) (H), granulocytes (CD11b<sup>+</sup>Gr1<sup>+</sup>) (I) and monocytes (CD11b<sup>+</sup>Gr1<sup>+</sup>F4/80<sup>+</sup>) (J) was analyzed using flow cytometry. Bars represent mean  $\pm$  Standard Error of Mean. \* $P < 0.05$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns: not significant.



**Figure 4. ASXL2 is essential for normal thymocyte maturation.** (A) Thymocyte count in >1-year old wild-type (WT) and *Asx2* knockout (KO) mice. (B) Representative FACS plots depict the proportion of DN (CD4<sup>-</sup>CD8<sup>-</sup>), DP (CD4<sup>+</sup>CD8<sup>+</sup>), CD4<sup>+</sup> single positive (SP) and CD8<sup>+</sup> SP cells in the thymus of old WT and *Asx2* KO mice. (C) Frequencies of DP, DN, CD4<sup>+</sup> SP and CD8<sup>+</sup> SP populations in old WT (n=17) and *Asx2* KO (n=18) mice. (D) FACS plots show representative flow cytometric staining for surface expression of CD44 and CD25 within the DN population in the thymus of old mice. (E) Proportions of DN1 (CD44<sup>+</sup>CD25<sup>-</sup> DN), DN2 (CD44<sup>+</sup>CD25<sup>+</sup> DN), DN3 (CD44<sup>-</sup>CD25<sup>+</sup> DN) and DN4 (CD44<sup>-</sup>CD25<sup>-</sup> DN) sub-populations within the DN compartment of thymus of old WT and KO mice. (F and G) Frequencies of myeloid cells (CD11b<sup>+</sup>) (F) and B cells (CD19<sup>+</sup>) (G) in the thymus of >1-year old WT and KO mice (n=5-7). Data are represented as mean±Standard Error of Mean. \*\*\*P<0.001, \*\*\*\*P<0.0001.

quency of CD11b<sup>+</sup> cells and myeloperoxidase-positive cells (Figure 2E and *Online Supplementary Figure S8B* and C). The absolute numbers of CD11b<sup>+</sup> myeloid cells were significantly increased (20-fold) while the number of lymphoid cells were 2-fold higher in the spleens of KO mice compared with the WT mice (Figure 2F). Histological examination of spleens demonstrated loss of normal architecture in KO mice compared with the WT mice (*data not shown*). Moreover, extramedullary hematopoiesis in the KO spleens was evident by an elevated proportion and numbers of total Lin<sup>+</sup>Kit<sup>+</sup> and Lin<sup>+</sup>Kit<sup>+</sup>Sca1<sup>+</sup> (LSK) cells (Figure 2G and *Online Supplementary Figure S9A-D*) as well as significantly higher frequency of erythroid progenitors (populations proE, EryA and EryB) (Figure 2H and *Online Supplementary Figure S9E*). We also detected markedly increased frequency of stem/progenitor cells in the peripheral blood of old KO mice (Figure 2G and *Online Supplementary Figure S9F*).

Parallel analyses of spleens from young KO mice (8-14 weeks old) revealed modestly increased spleen size with a trend towards elevated proportion and number of LSK cells (*Online Supplementary Figure S10A-C*). A propensity for increased frequencies of myeloid cells (CD11b<sup>+</sup>) and erythroid progenitors was also noted in the spleens of young KO mice (*Online Supplementary Figure S10D* and E). This indicates an onset of extramedullary hematopoiesis in young mice, which manifests by marked myeloid and erythroid cell expansion as the mice grow older.

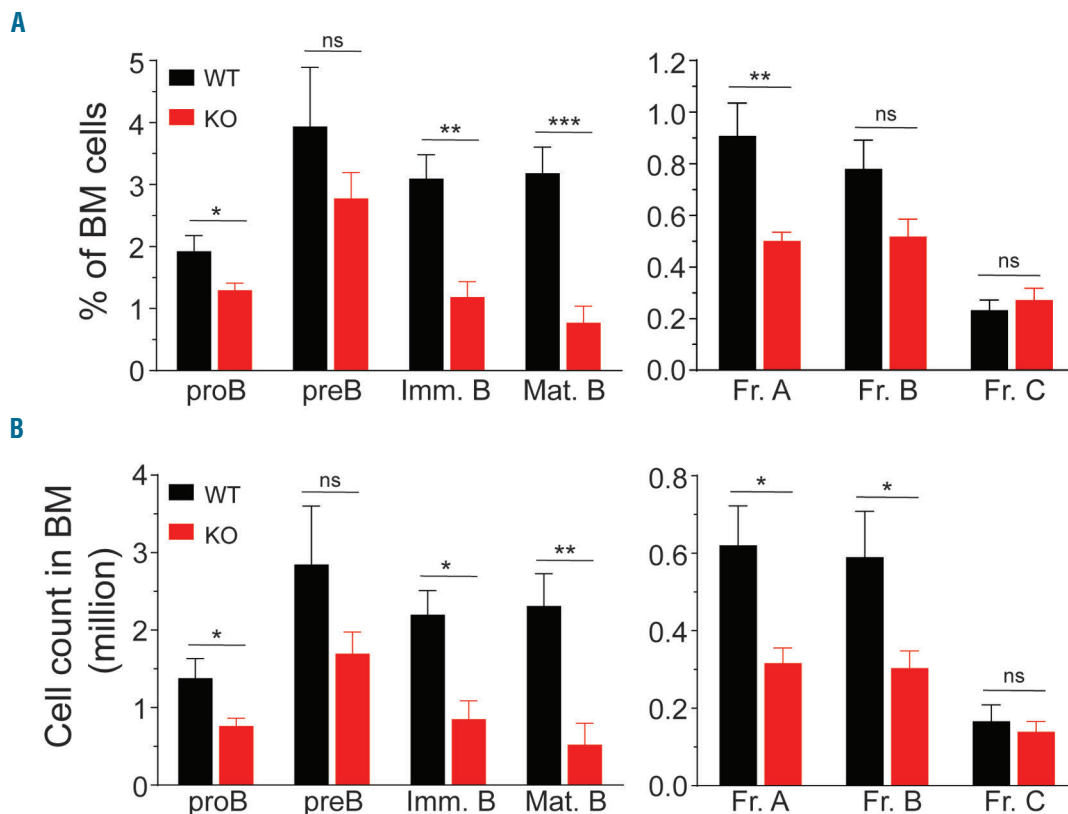
#### ASXL2 deficiency results in defective differentiation and function of hematopoietic stem cells

ASXL2 deficiency resulted in paler bones and decreased marrow cellularity in old mice (Figure 3A and B). Notably, BM of old *Asx2* KO mice had higher frequency and absolute number of LSK cells compared with the WT mice

(Figure 3C and *Online Supplementary Figure S11A* and B). The proportion of LT-HSCs (CD34<sup>+</sup>Flt3<sup>-</sup>LSK), ST-HSCs (CD34<sup>+</sup>Flt3<sup>+</sup>LSK) and MPPs (CD34<sup>+</sup>Flt3<sup>+</sup>LSK) within the LSK compartment was largely unaltered (*Online Supplementary Figure S11C*), albeit an overall increase in the absolute number of these subpopulations occurred in the old KO mice (*Online Supplementary Figure S11D* and E). We also observed reduced BM cellularity in young KO mice; and although the proportion of LSK cells was not significantly altered, the frequency and absolute numbers of LT-HSCs were significantly higher, suggesting early defects in maintaining HSC frequency (*Online Supplementary Figure S11F-J*). *In vivo* BrdU incorporation assay uncovered a significantly higher frequency of BrdU<sup>+</sup> LSK cells in the BM of *Asx2* KO mice, indicating increased cycling of stem/progenitor cells lacking ASXL2 (Figure 3D). These results illustrate that ASXL2 is required for maintaining the number and self-renewal of HSCs during steady-state hematopoiesis.

Flow cytometric analyses also demonstrated decreased proportion and number of common myeloid precursors (CMP; Lin<sup>+</sup>Kit<sup>+</sup>Sca1<sup>+</sup>CD34<sup>+</sup>FcyRII/III<sup>lo</sup>) and granulocyte monocyte precursors (GMP; Lin<sup>+</sup>Kit<sup>+</sup>Sca1<sup>+</sup>CD34<sup>+</sup>FcyRII/III<sup>hi</sup>) in >1-year old *Asx2* KO mice (Figure 3E and *Online Supplementary Figure S12A* and B). Furthermore, a marked reduction in the frequencies of erythroid precursors was noted in the old KO mice (Figure 3F and *Online Supplementary Figure S12C*), indicating impaired erythropoiesis. Reduction in erythroid precursors as well as CMP and GMP populations was also apparent in the BM of the young KO mice, signifying an early inception of defects in erythroid and myeloid differentiation because of ASXL2 deficiency (*Online Supplementary Figure S12D-F*).

Further, multi-lineage reconstitution ability of *Asx2*-deficient HSCs was assessed in a competitive repop-



**Figure 5. Impaired B-cell lymphopoiesis in *Asxl2*-deficient mice.** (A and B) Proportion (A) and absolute number (B) of cells at different stages of B-cell development in the bone marrow of >1-year old WT and *Asxl2* KO mice ( $n=4-6$ ). proB: CD43<sup>+</sup>B220<sup>+</sup>, preB: CD43<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup>, immature B (Imm. B): CD43<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup>, mature B (Mat. B): CD43<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup>, Fraction A (Fr. A): CD24<sup>+</sup>BP1<sup>+</sup> proB cells, Fraction B (Fr. B): CD24<sup>+</sup>BP1<sup>+</sup> proB cells, Fraction C (Fr. C): CD24<sup>+</sup>BP1<sup>+</sup> proB cells. Data are represented as mean±Standard Error of Mean. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , ns: not significant.

ulation assay. ASXL2 deficiency resulted in poor reconstitution of lymphoid lineage (both B and T cells) compared with the WT cells (Figure 3G and H). The reconstitution of granulocytes was also impaired in mice transplanted with *Asxl2*-deficient LSK cells (Figure 3I), while the proportion of donor-derived monocytes was similar to the recipients transplanted with WT cells (Figure 3J). These repopulation assays demonstrated that ASXL2 is essential for differentiation into multiple hematopoietic lineages including the lymphoid lineage, prompting us to investigate the development of lymphoid lineage in the *Asxl2* KO mice.

#### Impaired thymopoiesis in mice lacking ASXL2

A marked reduction of thymus size and cellularity was a consistent feature in >1-year old *Asxl2* KO mice compared with the WT mice (Figure 4A). Flow cytometric analyses revealed a striking reduction in CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) cells and a concomitant increase in the proportion and number of CD4<sup>+</sup>CD8<sup>+</sup> double negative (DN) cells in the thymi of *Asxl2* KO mice (Figure 4B and C and *Online Supplementary Figure S13A*). Within the DN compartment, the proportion of DN1 (CD44<sup>+</sup>CD25<sup>+</sup>) subpopulation was unchanged, whereas a significant reduction was detected in the proportion of DN2 (CD44<sup>+</sup>CD25<sup>+</sup>) and DN3 (CD44<sup>+</sup>CD25<sup>+</sup>) subpopulations, indicating a partial block in differentiation of earliest thymocytes (Figure 4D and E). A large number of DN cells in the thymi of KO mice consisted primarily of CD25<sup>+</sup> cells,

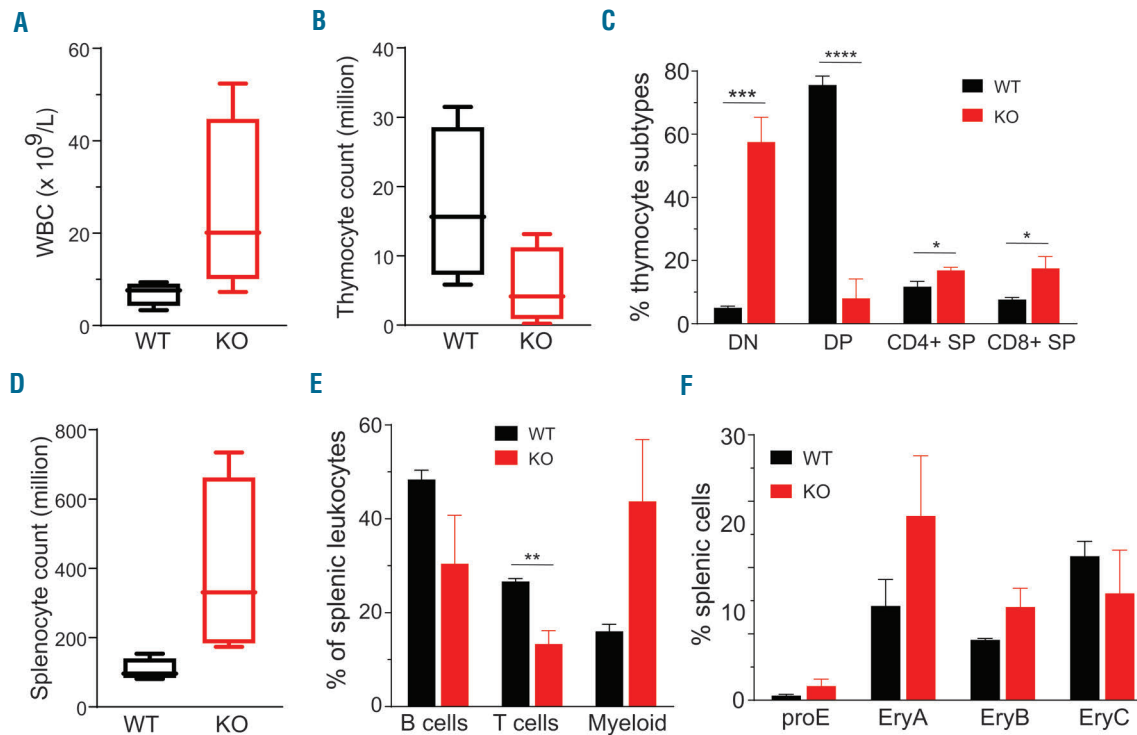
present in the DN1 and DN4 gates (Figure 4D and *Online Supplementary Figure S13B*). Further characterization showed that the vast majority of cells in the DN gate were CD19<sup>+</sup> (which contributed to 40% of total thymus-residing hematopoietic cells), and the frequency and number of CD11b<sup>+</sup> myeloid cells were also noticeably increased in the thymus of KO mice compared with the WT mice (Figure 4F and G and *Online Supplementary Figure S13C-F*).

Young *Asxl2* KO mice exhibited decreased thymus cellularity, but displayed less pronounced effect on major thymic compartments compared with the >1-year old mice (*Online Supplementary Figure S13G-I*). These findings illustrate that ASXL2 is essential for maintaining normal thymopoiesis in mice and its deficiency leads to progressive impairment in thymocyte maturation, which is manifested by a partial block from the DN to the DP stage, and an accumulation of myeloid and B cells in the thymus of the old mice.

#### Defects in B-cell development in *Asxl2* knockout mice

We also examined the consequences of ASXL2 deficiency on B-cell development in the BM by examining the expression of several surface antigens, which define the advancing stages of B-cell maturation. While the young *Asxl2* KO mice showed largely conserved subsets during B-cell lymphopoiesis, a notable decrease in the proportion of mature B cells (CD43<sup>+</sup>IgM<sup>+</sup>B220<sup>hi</sup>) compared with the WT mice was observed (*Online Supplementary Figure S14*).





**Figure 6. Cell-intrinsic effects of *Asxl2* deficiency on lymphoid and myeloid lineages.** Lethally irradiated mice transplanted with either wild-type (WT) or *Asxl2* knock-out (KO) bone marrow (BM) cells were analyzed after one year. (A) Number of white blood cells (WBCs) in the peripheral blood of recipient mice. (B) Number of thymocytes in recipient mice. (C) Proportion of donor-derived (CD45.2<sup>+</sup>) DN, DP, CD4<sup>+</sup>SP and CD8<sup>+</sup>SP cells in thymi of recipient mice. (D) Spleen cellularity. (E) Percentages of CD45.2<sup>+</sup> B, T and myeloid cells in the spleen of mice transplanted with either WT or KO cells. (F) Frequencies of erythroid precursors in spleens of recipient mice. Bars represent mean  $\pm$  Standard Error of Mean. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

However, the BM of old KO mice showed a substantial reduction in the frequencies and absolute number of proB (CD43<sup>+</sup>B220<sup>+</sup>), preB (CD43<sup>+</sup>IgM<sup>+</sup>B220<sup>+</sup>), immature B (CD43<sup>+</sup>IgM<sup>+</sup>B220<sup>+</sup>), and mature B cells (Figure 5A and B), indicating an age-dependent paucity in B-cell development. Further fractionation of proB cells using the scheme proposed previously,<sup>36</sup> revealed a lower proportion of CD24<sup>+</sup>BP1<sup>+</sup> (Fraction A) and CD24<sup>+</sup>BP1<sup>+</sup> (Fraction B) proB cells in the old *Asxl2* KO mice (Figure 5A and B), demonstrating a partial arrest of B-cell maturation.

#### Cell-intrinsic role of ASXL2 in hematopoiesis

To test the cell-autonomous function of ASXL2 in hematopoietic development, we transplanted lethally irradiated mice with either WT or *Asxl2* KO BM cells and analyzed the hematopoietic compartment one year later. We observed that the recipient mice transplanted with *Asxl2* KO BM cells tended to have higher WBC counts in peripheral blood (Figure 6A), similar to the age-dependent increase in WBC observed in KO mice (Figure 2A). We also noted decreased thymocyte cellularity and accumulation of donor-derived DN cells in the thymi of mice transplanted with *Asxl2* KO BM compared with those transplanted with WT BM (Figure 6B and C). Spleens of *Asxl2* KO BM-recipients were enlarged and exhibited elevated proportion of myeloid cells (Figure 6D and E). Moreover, our analysis also indicated a higher proportion of erythroid precursors in spleens of mice reconstituted with *Asxl2* KO BM cells compared with the WT cells (Figure 6F), similar to the phenotype observed in old *Asxl2* KO mice. Overall, the recipient mice recapitulated hematopoi-

etic features of old *Asxl2* KO mice, suggesting a hematopoietic cell-intrinsic function of ASXL2.

#### Identification of altered gene expression in ASXL2-deficient hematopoietic stem cells

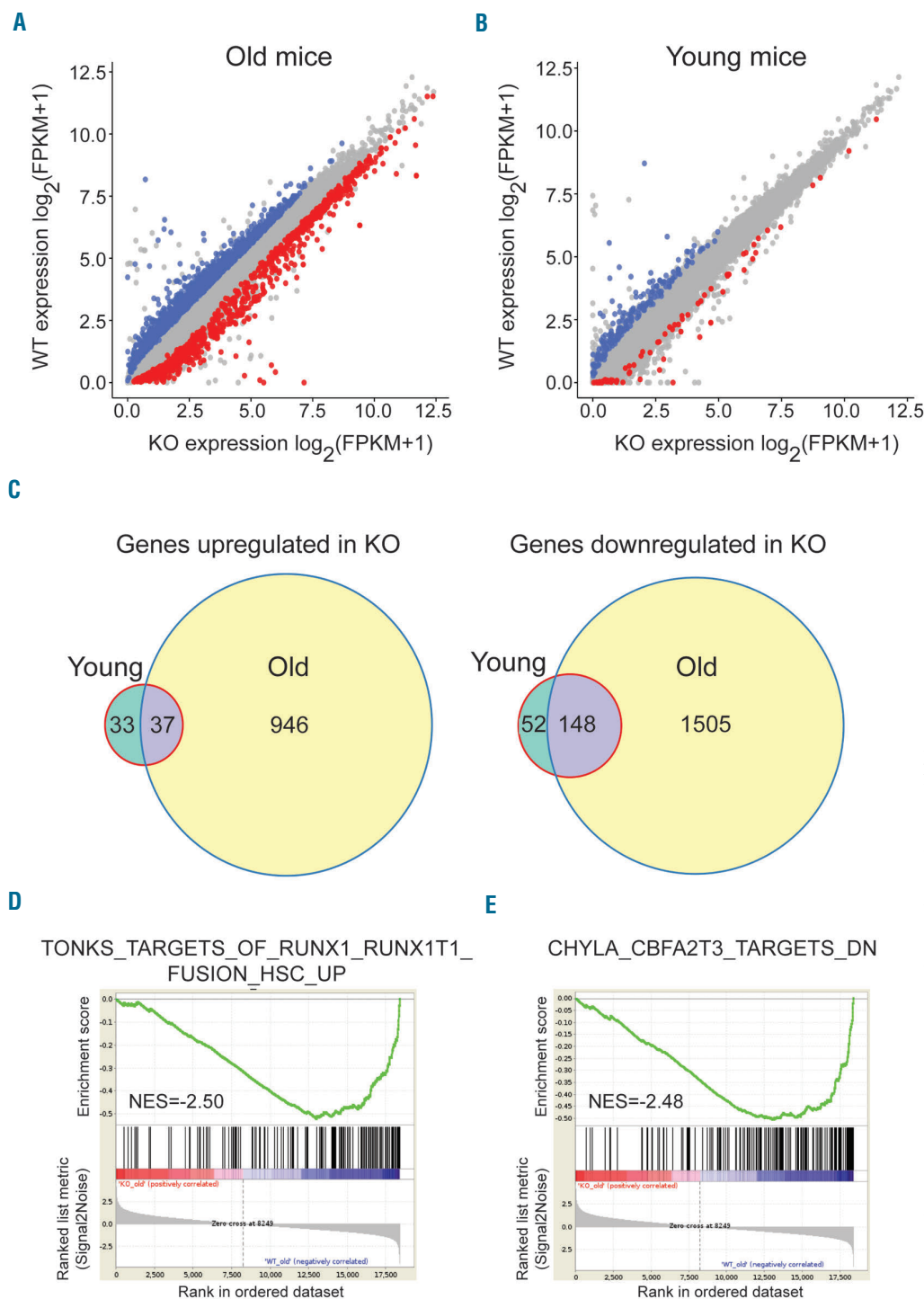
To determine the molecular basis of the defects observed in *Asxl2* KO HSCs, we performed global gene expression profiling (RNA-Sequencing) of sorted LSK cells from old and young WT and *Asxl2* KO mice. Comparison of transcriptomic profiles of LSK cells from old WT and *Asxl2* KO identified more than 2500 genes differentially expressed (983 genes up-regulated and 1653 genes down-regulated in the KO cells; FDR < 0.1) (Online Supplementary Table S6), including those involved in myeloid differentiation such as *Csf1*, *Gfi1b*, *Gata2*, *Hoxa9*, *Hoxa5*, *Mpl*, *Cdk6*, *Ccr1* and *Ets1* (Online Supplementary Figure S15A). *Asxl2*-deficient LSK cells from old mice exhibited more pronounced changes in gene expression compared with the young mice (Figure 7A and B and Online Supplementary Figure S15B). However, a significant overlap of genes commonly up-regulated and down-regulated in the KO LSK cells was noted within the two age groups (Figure 7C and Online Supplementary Table S6). Interestingly, GSEA analysis revealed that the expression of RUNX1-RUNX1T1 targets<sup>37</sup> inversely correlated in the KO cells (Figure 7D and Online Supplementary Figure S15C). Also, genes down-regulated in immature BM progenitors upon silencing of *CBFA2T3* (also called *ETO2*)<sup>38</sup> were also suppressed in the LSK cells from both old and young *Asxl2* KO mice (Figure 7E and Online Supplementary Figure S15D). *CBFA2T3* is involved in the translocation t(16;21)(q24;q22) with

*RUNX1* in AML, a disease with phenotypic and transcriptional profile reminiscent of t(8;21) AML.<sup>39,40</sup>

## Discussion

This study, along with recent reports,<sup>5-10</sup> provides a comprehensive landscape of the mutational spectrum of t(8;21) AML, which is distinctive from other subtypes of AML and characterized by high frequency of alterations of *KIT*, *ASXL2*, *DHX15* and *MGA* genes; and rare mutations

of other commonly altered genes in myeloid leukemia (*DNMT3A*, *RUNX1*, *IDH2*, *NPM1*). Truncating mutations of *ASXL2* occurred in 20% of newly-diagnosed t(8;21) AML cases, and were localized to exons 11 and 12, a pattern highly reminiscent of the mutational profile for *ASXL1* in hematologic malignancies.<sup>41,42</sup> However, unlike *ASXL1*, which is altered in a diverse range of hematologic diseases, mutations of *ASXL2* are specific to the t(8;21) AML subtype. We also noted that the mutational frequency of RAS pathway genes (*NRAS* and *KRAS*) and *ZBTB7A* in our Asian cohort of t(8;21) AML were notably lower



**Figure 7. Gene expression changes in *Asxl2*-deficient LSK cells.** (A and B) Changes in transcript levels in LSK cells from >1-year old (A) and young (8-12-week old) (B) *Asxl2* knockout (KO) versus wild-type (WT) littermates. For each age group and genotype, cells from 2 mice were analyzed. Genes significantly up-regulated in KO cells are represented in red circles while down-regulated genes are shown in blue (FDR<0.1). (C) Venn diagrams show overlap of genes either up-regulated or down-regulated in young and old *Asxl2* KO LSK cells compared with the WT cells from young and old mice, respectively. (D and E) GSEA comparing expression of genes in LSK cells from >1-year old WT and KO littermates for the selected gene sets (FDR<0.1): genes up-regulated in normal hematopoietic progenitors by RUNX1-RUNX1T1 fusion (D), and genes down-regulated in hematopoietic progenitors in CBFA2T3 KO mice (E). NES: normalized enrichment score.

than previous reports from Western populations.<sup>5,7-9,40,43</sup> We obtained a mutational rate of 6% for *NRAS* and 2% for *KRAS* compared to 13-26% for *NRAS* and 4-7% for *KRAS* observed in previous studies.<sup>5,7-9,43</sup> Similarly, *ZBTB7A* mutations that are frequent in Caucasian t(8;21) AML (9-23%)<sup>9,40,43</sup> were observed in only 3% of our Asian patient cohort. Although this might reflect a true difference between genetic backgrounds, this requires further confirmation in independent t(8;21) AML cohorts from different genetic backgrounds.

In this study, we focused on functional characterization of *Asxl2* in hematopoietic differentiation using a mouse model of *Asxl2* deficiency. Mice used in this study were C57BL/6 x 129Sv F1 as the animals in either C57BL/6 or 129Sv background die perinatally,<sup>23</sup> which precludes investigation of *Asxl2* knockout on an inbred genetic background. Moreover, this mouse model exhibits constitutive loss of *Asxl2*, which is distinct from the truncating somatic mutations of *ASXL2* observed in t(8;21) AML that may possibly generate a C-terminal truncated protein. Despite these limitations, our study strengthens the findings concerning the key role of *ASXL2* in hematopoiesis reported in two recent studies.<sup>27,28</sup> Furthermore, our study reports several additional features of *Asxl2* deficient mice, which had not been described previously. While the significance of *ASXL2* in maintaining normal hematopoiesis is evidenced from these studies, we performed a parallel in-depth analysis in young (8-14 weeks old) *versus* old (>1-year old) mice, which helped establish the progressive phenotype associated with *Asxl2* deficiency, including uncovering the crucial function of *ASXL2* in development of the lymphoid lineage. Age-dependent defects in hematopoiesis in the knockout mice were characterized by an increased proportion of LSK cells and reduced frequencies of CMP and GMP cells in the BM of >1-year old KO mice. *Asxl2*-deficient mice also exhibited a progressive myeloproliferative phenotype, accompanied by increased peripheral WBC counts, splenomegaly and extramedullary hematopoiesis, indicating perturbed hematopoiesis. In addition, erythroid maturation was impaired in the old mice lacking *ASXL2*, signifying age-dependent defects in erythroid development. Both previous studies showed that *ASXL2* deficiency led to poor reconstitution ability of HSCs in transplantation models, consistent with our findings. We observed that *Asxl2*-deficient LSK cells exhibited poor reconstitution ability of the lymphoid lineage and flow cytometric analyses of >1-year old mice demonstrated defects in both T-cell maturation in the thymus and B-cell development in the BM of the old KO mice. Apart from the intrinsic effect of *ASXL2* on lymphoid differentiation, chronic overproduction of myeloid cells and accompanying inflammatory signals in the mice lacking *ASXL2* may also negatively affect the lymphoid output. These findings establish *ASXL2* as an essential

component of hematopoietic development in mice.

Gene expression analysis of sorted LSK cells from WT and *Asxl2* KO BM identified several key regulators of hematopoiesis as downstream targets of *ASXL2* in HSCs. Prominently, GSEA analysis revealed that the expression of genes regulated by RUNX1-RUNX1T1 correlated with the expression of *ASXL2*, indicating *ASXL2* is possibly required for transcriptional activity of RUNX1-RUNX1T1. An identical finding was described recently for *Asxl2*-deficient LSK cells in an independent mouse model. Here the authors also detected binding of *ASXL2* to the genomic loci occupied by RUNX1 and RUNX1-RUNX1T1, although no direct interaction between *ASXL2* and either RUNX1 or RUNX1-RUNX1T1 was observed.<sup>27</sup> Further studies are needed to investigate whether *ASXL2* acts as a likely co-regulator of RUNX1-RUNX1T1 during leukemogenesis. GSEA also indicated a correlation between *Asxl2* deficiency and the expression levels of reported targets of BMI1 (a PRC1 member)<sup>44</sup> and SUZ12 (a PRC2 member)<sup>45</sup> (Online Supplementary Figure S15E and F), consistent with a role for *ASXL2* in transcriptional regulation mediated through the PRC complexes.<sup>12</sup>

In summary, the current study identified frequent *ASXL2* mutations in t(8;21) AML and characterized its role in hematopoietic development in mice. This work demonstrates that *ASXL2* plays a critical role in multi-lineage differentiation and highlights how its loss leads to progressive hematopoietic defects and promotes myeloid expansion, thereby advancing our understanding of epigenetic machinery that regulates hematopoiesis.

### Acknowledgments

We would like to thank the staff of Comparative Medicine, NUS for their support in mice maintenance and experiments. We would also like to acknowledge expert help and support from the FACS facility at CSI, Singapore. We also appreciate the help of Dr. Motomi Osato, CSI, Singapore, for providing reagents and Dr. Maya Jeitany for critical reading of the manuscript and useful discussions. We thank the Melamed Family and Reuben Yeroushalmi for their generous support.

### Funding

This work was funded by the Leukemia and Lymphoma Society, the Singapore Ministry of Health's National Medical Research Council (NMRC) under its Singapore Translational Research (STaR) Investigator Award to HPK (NMRC/STaR/0021/2014), Singapore Ministry of Education Academic Research Fund Tier 2 (MOE2013-T2-2-150), the NMRC Centre Grant awarded to National University Cancer Institute of Singapore (NMRC/CG/012/2013) and the National Research Foundation Singapore and the Singapore Ministry of Education under its Research Centres of Excellence initiatives. The study was supported by grant MOHW104-TDU-B-212-124-006, OMRPG3C0021 awarded to L-YS.

### References

- Downing JR. The AML1-ETO chimaeric transcription factor in acute myeloid leukaemia: biology and clinical significance. *Br J Haematol*. 1999;106(2):296-308.
- Peterson LF, Boyapati A, Ahn EY, et al. Acute myeloid leukemia with the 8q22;21q22 translocation: secondary mutational events and alternative t(8;21) transcripts. *Blood*. 2007;110(3):799-805.
- Hatlen MA, Wang L, Nimer SD. AML1-ETO driven acute leukemia: insights into pathogenesis and potential therapeutic approaches. *Front Med*. 2012;6(3):248-262.
- Muller AM, Duque J, Shizuru JA, Lubbert M. Complementing mutations in core binding factor leukemias: from mouse models to clinical applications. *Oncogene*. 2008;27(44):5759-5773.
- Micol JB, Duployez N, Boissel N, et al. Frequent *ASXL2* mutations in acute myeloid leukemia patients with t(8;21)/RUNX1-RUNX1T1 chromosomal translocations. *Blood*. 2014;124(9):1445-1449.
- Sood R, Hansen NE, Donovan FX, et al.



- Somatic mutational landscape of AML with inv(16) or t(8;21) identifies patterns of clonal evolution in relapse leukemia. *Leukemia*. 2016;30(2):501-504.
7. Krauth MT, Eder C, Alpermann T, et al. High number of additional genetic lesions in acute myeloid leukemia with t(8;21)/RUNX1-RUNX1T1: frequency and impact on clinical outcome. *Leukemia*. 2014;28(7):1449-1458.
  8. Duployez N, Marceau-Renaut A, Boissel N, et al. Comprehensive mutational profiling of core binding factor acute myeloid leukemia. *Blood*. 2016;127(20):2451-2459.
  9. Faber ZJ, Chen X, Gedman AL, et al. The genomic landscape of core-binding factor acute myeloid leukemias. *Nat Genet*. 2016; 48(12):1551-1556.
  10. Yamato G, Shiba N, Yoshida K, et al. ASXL2 Mutations are Frequently Found in Pediatric AML Patients with t(8;21)/RUNX1-RUNX1T1 and Associated with a Better Prognosis. *Genes Chromosomes Cancer*. 2017;56(5):382-393.
  11. Hatlen MA, Arora K, Vacic V, et al. Integrative genetic analysis of mouse and human AML identifies cooperating disease alleles. *J Exp Med*. 2016;213(1):25-34.
  12. Katoh M. Functional and cancer genomics of ASXL family members. *Br J Cancer*. 2013;109(2):299-306.
  13. Katoh M. Functional proteomics of the epigenetic regulators ASXL1, ASXL2 and ASXL3: a convergence of proteomics and epigenetics for translational medicine. *Expert Rev Proteomics*. 2015;12(3):317-328.
  14. Haeflrich T, Nagata Y, Grossmann V, et al. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia*. 2014;28(2):241-247.
  15. Carubbia N, Murati A, Trouplin V, et al. Mutations of ASXL1 gene in myeloproliferative neoplasms. *Leukemia*. 2009; 23(11):2183-2186.
  16. Quesada V, Conde L, Villamor N, et al. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. *Nat Genet*. 2012;44(1):47-52.
  17. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*. 2013;368(22):2059-2074.
  18. Yoshizato T, Dumitriu B, Hosokawa K, et al. Somatic Mutations and Clonal Hematopoiesis in Aplastic Anemia. *N Engl J Med*. 2015;373(1):35-47.
  19. Abdel-Wahab O, Pardanani A, Patel J, et al. Concomitant analysis of EZH2 and ASXL1 mutations in myelofibrosis, chronic myelomonocytic leukemia and blast-phase myeloproliferative neoplasms. *Leukemia*. 2011;25(7):1200-1202.
  20. Boultonwood J, Perry J, Zaman R, et al. High-density single nucleotide polymorphism array analysis and ASXL1 gene mutation screening in chronic myeloid leukemia during disease progression. *Leukemia*. 2010;24(6):1139-1145.
  21. Abdel-Wahab O, Gao J, Adli M, et al. Deletion of Asxl1 results in myelodysplasia and severe developmental defects in vivo. *J Exp Med*. 2013;210(12):2641-2659.
  22. Farber CR, Bennett BJ, Orozco L, et al. Mouse genome-wide association and systems genetics identify Asxl2 as a regulator of bone mineral density and osteoclastogenesis. *PLoS Genet*. 2011;7(4):e1002038.
  23. Lai HL, Grachoff M, McGinley AL, et al. Maintenance of adult cardiac function requires the chromatin factor Asxl2. *J Mol Cell Cardiol*. 2012;53(5):734-741.
  24. Baskind HA, Na L, Ma Q, Patel MF, Geenen DL, Wang QT. Functional conservation of Asxl2, a murine homolog for the Drosophila enhancer of trithorax and polycomb group gene Asx. *PLoS One*. 2009;4(3):e4750.
  25. McGinley AL, Li Y, Deliu Z, Wang QT. Additional sex combs-like family genes are required for normal cardiovascular development. *Genesis*. 2014;52(7):671-686.
  26. Lai HL, Wang QT. Additional sex combs-like 2 is required for polycomb repressive complex 2 binding at select targets. *PLoS One*. 2013;8(9):e73983.
  27. Micol JB, Pastore A, Inoue D, et al. ASXL2 is essential for haematopoiesis and acts as a haploinsufficient tumour suppressor in leukemia. *Nat Commun*. 2017;8(15429).
  28. Li J, He F, Zhang P, et al. Loss of Asxl2 leads to myeloid malignancies in mice. *Nat Commun*. 2017;8:15456.
  29. Madan V, Shyamsunder P, Han L, et al. Comprehensive mutational analysis of primary and relapse acute promyelocytic leukemia. *Leukemia*. 2016;30(12):2430.
  30. Faust GG, Hall IM. SAMBLASTER: fast duplicate marking and structural variant read extraction. *Bioinformatics*. 2014; 30(17):2503-2505.
  31. Koboldt DC, Zhang Q, Larson DE, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res*. 2012; 22(3):568-576.
  32. McLaren W, Gil L, Hunt SE, et al. The Ensembl Variant Effect Predictor. *Genome Biol*. 2016;17(1):122.
  33. Cibulskis K, Lawrence MS, Carter SL, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol*. 2013;31(3):213-219.
  34. Mayakonda A, Koeffler HP. Maftools: Efficient analysis, visualization and summarization of MAF files from large-scale cohort based cancer studies. *bioRxiv*. 2016 May 11. Available from: <http://doi.org/10.1101/052662>
  35. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481(7382):506-510.
  36. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med*. 1991;173(5):1213-1225.
  37. Tonks A, Pearn L, Musson M, et al. Transcriptional dysregulation mediated by RUNX1-RUNX1T1 in normal human progenitor cells and in acute myeloid leukaemia. *Leukemia*. 2007;21(12):2495-2505.
  38. Chyla BJ, Moreno-Miralles I, Steapleton MA, et al. Deletion of Mtg16, a target of t(16;21), alters hematopoietic progenitor cell proliferation and lineage allocation. *Mol Cell Biol*. 2008;28(20):6234-6247.
  39. Athanasiadou A, Stalika E, Sidi V, Papaioannou M, Gaitatzi M, Anagnostopoulos A. RUNX1-MTG16 fusion gene in de novo acute myeloblastic leukemia with t(16;21)(q24;q22). *Leuk Lymphoma*. 2011;52(1):145-147.
  40. Lavalley VP, Lemieux S, Boucher G, et al. RNA-sequencing analysis of core binding factor AML identifies recurrent ZBTB7A mutations and defines RUNX1-CBFA2T3 fusion signature. *Blood*. 2016;127(20):2498-2501.
  41. Gelsi-Boyer V, Brecqueville M, Devillier R, Murati A, Mozziconacci MJ, Birnbaum D. Mutations in ASXL1 are associated with poor prognosis across the spectrum of malignant myeloid diseases. *J Hematol Oncol*. 2012;5:12.
  42. Schnittger S, Eder C, Jeromin S, et al. ASXL1 exon 12 mutations are frequent in AML with intermediate risk karyotype and are independently associated with an adverse outcome. *Leukemia*. 2013;27(1):82-91.
  43. Hartmann L, Dutta S, Opatz S, et al. ZBTB7A mutations in acute myeloid leukaemia with t(8;21) translocation. *Nat Commun*. 2016;7:11733.
  44. Douglas D, Hsu JH, Hung L, et al. BMI-1 promotes ewing sarcoma tumorigenicity independent of CDKN2A repression. *Cancer Res*. 2008;68(16):6507-6515.
  45. Pasini D, Bracken AP, Hansen JB, Capillo M, Helin K. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. *Mol Cell Biol*. 2007; 27(10):3769-3779.